NEBNext® ARTIC SARS-CoV-2 RT-PCR Module

NEB #E7626S/L

24/96 reactions

Version 2.0_9/21

Table of Contents

The Library Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7626S) and 96 reactions (NEB #E7626L). Colored bullets represent the color of the cap of the tube containing the reagent.

Package 1: Store at –20°C.

- (lilac) LunaScript® RT SuperMix (5X)
- (lilac) Q5® Hot Start High-Fidelity 2X Master Mix
- (lilac) NEBNext ARTIC SARS-CoV-2 Primer Mix 1
- (lilac) NEBNext ARTIC SARS-CoV-2 Primer Mix 2
- (orange) NEBNext VarSkip Short SARS-CoV-2 Primer Mix 1
- (orange) NEBNext VarSkip Short SARS-CoV-2 Primer Mix 2
- (white) 0.1X TE
- (white) Nuclease-free Water

Required Materials Not Included

- 80% Ethanol (freshly prepared)
- Nuclease-free Water
- DNA LoBind Tubes (Eppendorf® #022431021)
- Magnetic rack/stand (NEB #S1515, Alpaqua®, cat. #A001322 or equivalent)
- Thermal cycler
- Vortex Mixer
- Microcentrifuge
- Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables
- DNase RNase free PCR strip tubes (USA Scientific® 1402-1708)

Protocol Descriptions

Chapter 1: cDNA Synthesis and Targeted cDNA Amplification with NEBNext ARTIC Primer Mixes: This protocol utilizes NEBNext ARTIC SARS-CoV-2 Primer Mixes for targeting SARS-CoV-2, these are balanced ARTICv3 primers. If downstream applications include sequencing, performing RNA input normalization prior to cDNA synthesis and targeted amplification promotes more even distribution of reads across sequencing libraries.

Chapter 2: cDNA Synthesis and Targeted cDNA Amplification with NEBNext VarSkip Short Primer Mixes: This protocol follows an alternate variant-tolerant approach for targeting SARS-CoV-2 by utilizing NEBNext VarSkip Short SARS-CoV-2 Primer Mixes. The NEBNext VarSkip Short SARS-CoV-2 Primer mixes cannot be added to the same cDNA amplification reaction as the NEBNext ARTIC SARS-CoV-2 Primer Mixes. If downstream applications include sequencing, performing RNA input normalization prior to cDNA synthesis and targeted amplification promotes more even distribution of reads across sequencing libraries.

Overview

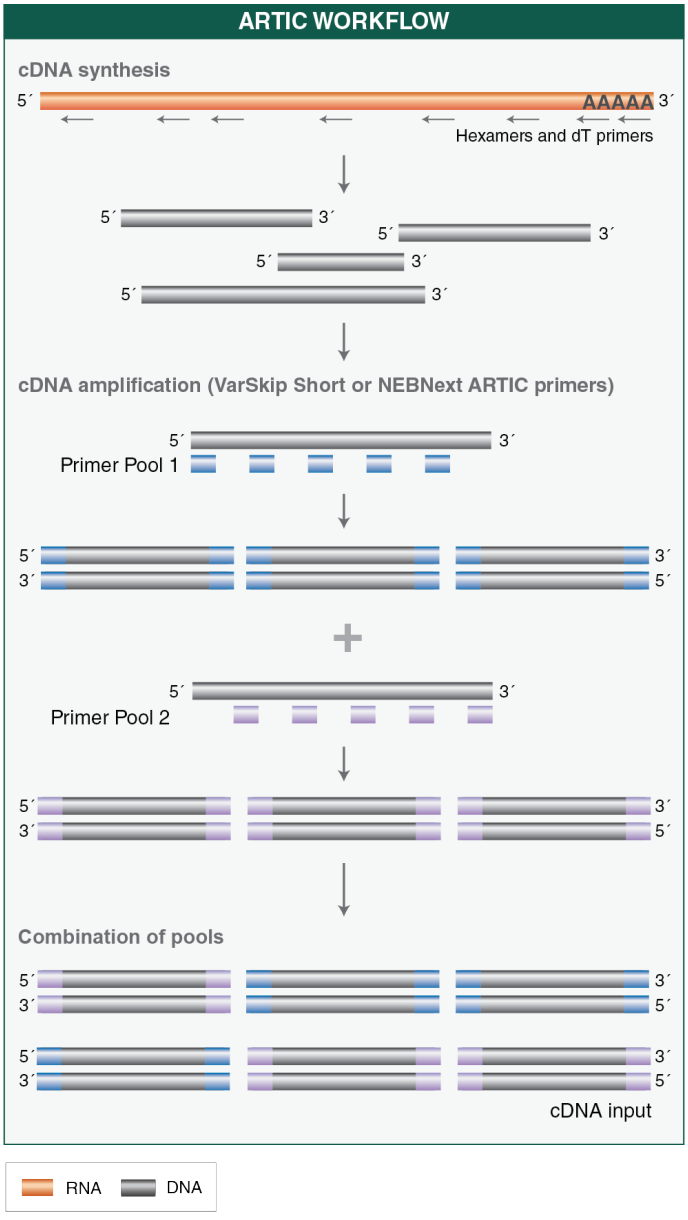
The NEBNext ARTIC SARS-CoV-2 RT-PCR Module contains the enzymes and buffers required to convert a broad range of total RNA into high quality, targeted cDNA amplicons with minimal hands-on time.

The NEBNext VarSkip Short SARS-CoV-2 Primer Mixes included provide an alternate variant-tolerant approach for targeting SARS-CoV-2. The NEBNext VarSkip Short SARS-CoV-2 Primer Mixes cannot be added to the same cDNA amplification reaction as the NEBNext ARTIC SARS-CoV-2 Primer Mixes.

Each kit component must pass rigorous quality control standards, and for each new lot the entire set of reagents is functionally validated together by construction and sequencing of indexed libraries on an Illumina® sequencing platform.

For larger volume requirements, customized and bulk packaging is available by purchasing through the OEM/Bulks department at NEB. Please contact OEM@neb.com for further information.

Figure 1. Workflow demonstrating the use of NEBNext ARTIC SARS-CoV-2 RT-PCR Module.



Chapter 1
cDNA Synthesis and Targeted cDNA Amplification with NEBNext ARTIC Primer Mixes

Symbols

This is a point where you can safely stop the protocol.

This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.

Colored bullets indicate the cap color of the reagent to be added to a reaction.

Note: The amount of RNA required for detection depends on the abundance of the RNA of interest. In general, we recommend using ≥ 10 copies of the SARS-CoV-2 viral genome as input, however, results may vary depending on the quality of the input. In addition, we recommend setting up a no template control reaction and that reactions are set-up in a hood.

1.1. cDNA Synthesis

The presence of carry-over products can interfere with sequencing accuracy, particularly for low copy targets. Therefore, it is important to carry out the appropriate no template control (NTC) reactions to demonstrate that positive reactions are meaningful.

1.1.1. Gently mix and spin down the LunaScript RT SuperMix reagent. Prepare the cDNA synthesis reaction as described below:

COMPONENT	VOLUME
RNA Sample	8 µl
● (lilac) LunaScript RT SuperMix	2 µl
Total Volume	10 µl

For no template controls, mix the following components:

COMPONENT	VOLUME
○ (white) Nuclease-free Water	8 µl
● (lilac) LunaScript RT SuperMix	2 µl
Total Volume	10 µl

1.1.2. Incubate reactions in a thermal cycler with the following steps:

CYCLE STEP	TEMP	TIME
Primer Annealing	25°C	2 minutes
cDNA Synthesis	55°C	20 minutes
Heat Inactivation	95°C	1 minute
Hold	4°C	∞

*Set heated lid to 105°C

Samples can be stored at -20°C for up to a week.

1.2. cDNA Amplification

Note: 4.5 µl cDNA input is recommended. If using less than 4.5 µl of cDNA, add nuclease-free water to a final volume of 4.5 µl. We recommend setting up the cDNA synthesis and cDNA amplification reactions in different rooms to minimize cross-contamination of future reactions.

1.2.1. Gently mix and spin down reagents. Prepare the split pool cDNA amplification reactions as described below:

For Pool Set A:

COMPONENT	VOLUME
cDNA (Step 1.1.2)	4.5 µl
• (lilac) Q5 Hot Start High-Fidelity 2X Master Mix	6.25 µl
ARTIC SARS-CoV-2 Primer Mix 1	1.75 µl
Total Volume	12.5 µl

For Pool Set B:

COMPONENT	VOLUME
cDNA (Step 1.1.2)	4.5 µl
• (lilac) Q5 Hot Start High-Fidelity 2X Master Mix	6.25 µl
ARTIC SARS-CoV-2 Primer Mix 2	1.75 µl
Total Volume	12.5 µl

1.2.2. Incubate reactions in a thermal cycler* with the following steps:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denature	95°C	15 seconds	35
Annealing/Extension	63°C	5 minutes	
Hold	4°C	∞	1

*Set heated lid to 105°C

1.2.3. Combine the Pool A and Pool B PCR reactions for each sample.

1.2.4. **Samples can be stored at -20°C for up to a week.**

Note: Please consider the downstream application before proceeding. Section 1.3 lists instructions for a standard bead cleanup, however, it may not be necessary for your application.

1.3. Cleanup of cDNA Amplicons.

Note: The volume of NEBNext Sample Purification Beads provided here are for use with the sample composition at this step (25 µl; Step 1.2.3). These bead volumes may not work properly for a cleanup at a different step in the workflow, or if this is a second cleanup at this step. For cleanups of samples contained in different buffer conditions, the volumes may need to be experimentally determined.

1.3.1. Vortex NEBNext Sample Purification Beads to resuspend.

1.3.2. Add 20 µl (0.8X) resuspended beads to the combined PCR reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

1.3.3. Incubate samples at room temperature for at least 5 minutes.

1.3.4. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant.

1.3.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard the beads)**.

1.3.6. Add 200 µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

1.3.7. Repeat Step 1.3.6. once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

1.3.8. Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.

1.3.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 16 µl 0.1X TE. Elution volume can be adjusted for specific applications.

1.3.10. Mix well by pipetting up and down 10 times, or vortexing 3-5 seconds on high. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

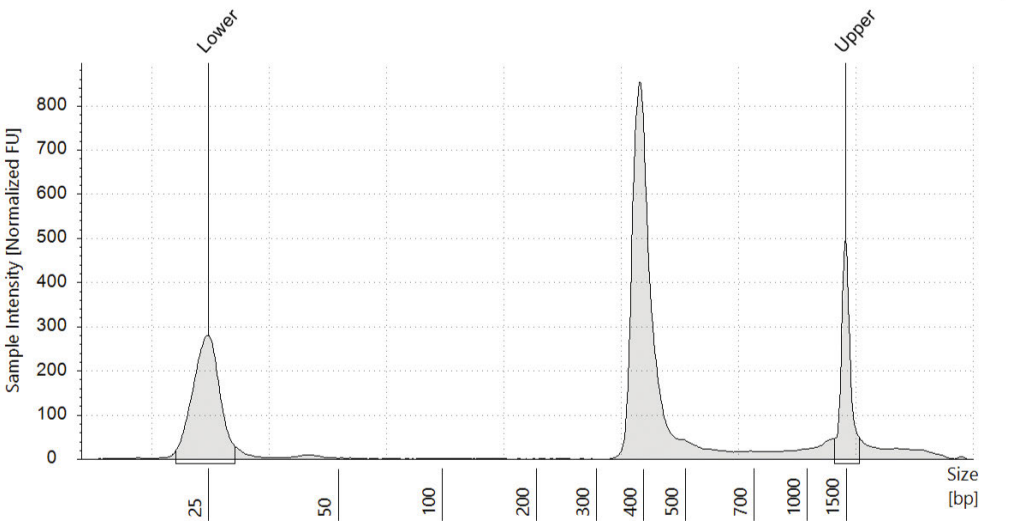
1.3.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 14 µl to a new PCR tube. If elution volume was adjusted in step 1.3.9, transfer volume should also be adjusted.

1.3.12. We recommend assessing cDNA amplicon (from Step 1.3.11) concentrations with a Qubit® fluorometer.

Note: Amplicons may also be run on a Bioanalyzer or TapeStation® to confirm 400 bp size of amplicons. To run on a TapeStation, dilute amplicon 10-fold with 0.1X TE Buffer and run 2 µl on a DNA High Sensitivity ScreenTape®. (See Figure 1.3.12. below for example of amplicon size profile on a Bioanalyzer).

Samples can be stored at –20°C for up to a week.

Figure 1.3.12: ARTIC SARS-CoV-2 cDNA amplicons generated from 1000 total viral copies.



Chapter 2

cDNA Synthesis and Targeted cDNA Amplification with NEBNext VarSkip Short Primer Mixes

Symbols

This is a point where you can safely stop the protocol.

This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.

- *Colored bullets indicate the cap color of the reagent to be added to a reaction.*

Note: The amount of RNA required for detection depends on the abundance of the RNA of interest. In general, we recommend, using ≥ 10 copies of the SARS-CoV-2 viral genome as input. In addition, we recommend setting up a no template control reaction and that reactions are set-up in a hood.

2.1. cDNA Synthesis

The presence of carry-over products can interfere with sequencing accuracy, particularly for low copy targets. Therefore, it is important to carry out the appropriate no template control (NTC) reactions to demonstrate that positive reactions are meaningful.

2.1.1. Gently mix and spin down the LunaScript RT SuperMix reagent. Prepare the cDNA synthesis reaction as described below:

COMPONENT	VOLUME
RNA Sample	8 µl
• (lilac) LunaScript RT SuperMix	2 µl
Total Volume	10 µl

For no template controls, mix the following components

COMPONENT	VOLUME
○ (white) Nuclease-free Water	8 µl
• (lilac) LunaScript RT SuperMix	2 µl
Total Volume	10 µl

2.1.2. Incubate reactions in a thermal cycler with lid temperature at 105°C with the following steps:

CYCLE STEP	TEMP	TIME
Primer Annealing	25°C	2 minutes
cDNA Synthesis	55°C	20 minutes
Heat Inactivation	95°C	1 minute
Hold	4°C	∞

*Set heated lid to 105°C

Samples can be stored at –20°C for up to a week.

2.2. cDNA Amplification

Note: 4.5 µl cDNA input is recommended. If using less than 4.5 µl of cDNA, add nuclease-free water to a final volume of 4.5 µl. We recommend setting up the cDNA synthesis and cDNA amplification reactions in different rooms to minimize cross-contamination of future reactions.

2.2.1. Gently mix and spin down reagents. Prepare the split pool amplification reactions as described below.

For Pool Set A:

COMPONENT	VOLUME
cDNA (Step 2.1.2)	4.5 µl
• (lilac) Q5 Hot Start High-Fidelity 2X Master Mix	6.25 µl
NEBNext VarSkip Short SARS-CoV-2 Primer Mix 1	1.75 µl
Total Volume	12.5 µl

For Pool Set B:

COMPONENT	VOLUME
cDNA (Step 2.1.2)	4.5 µl
• (lilac) Q5 Hot Start High-Fidelity 2X Master Mix	6.25 µl
NEBNext VarSkip Short SARS-CoV-2 Primer Mix 2	1.75 µl
Total Volume	12.5 µl

2.2.2. Incubate reactions in a thermal cycler* with the following steps:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 second	1
Denature	95°C	15 seconds	35
Annealing and Extension	63°C	5 minutes	
Hold	4°C	∞	1

* Set heated lid to 105°C.

2.2.3. Combine the Pool A and Pool B PCR reactions for each sample.

Samples can be stored at –20°C for up to a week.

Note: Please consider the downstream application before proceeding. Section 2.3 lists instructions for a standard bead cleanup, however, it may not be necessary for your application.

2.3. Cleanup of cDNA Amplicons

Note: The volume of NEBNext Sample Purification Beads provided here are for use with the sample composition at this step (25 µl; Step 2.2.3). These bead volumes may not work properly for a cleanup at a different step in the workflow, or if this is a second cleanup at this step. For cleanups of samples contained in different buffer conditions, the volumes may need to be experimentally determined.

2.3.1. Vortex NEBNext Sample Purification Beads to resuspend.

2.3.2. Add 20 µl (0.8X) resuspended beads to the combined PCR reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 3–5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

2.3.3. Incubate samples at room temperature for at least 5 minutes.

2.3.4. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant.

2.3.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard the beads)**.

2.3.6. Add 200 µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

2.3.7. Repeat Step 2.3.6. once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

2.3.8. Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.

2.3.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 16 µl 0.1X TE. Elution volume can be adjusted for specific applications.

2.3.10. Mix well by pipetting up and down 10 times, or vortexing 3-5 seconds on high. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

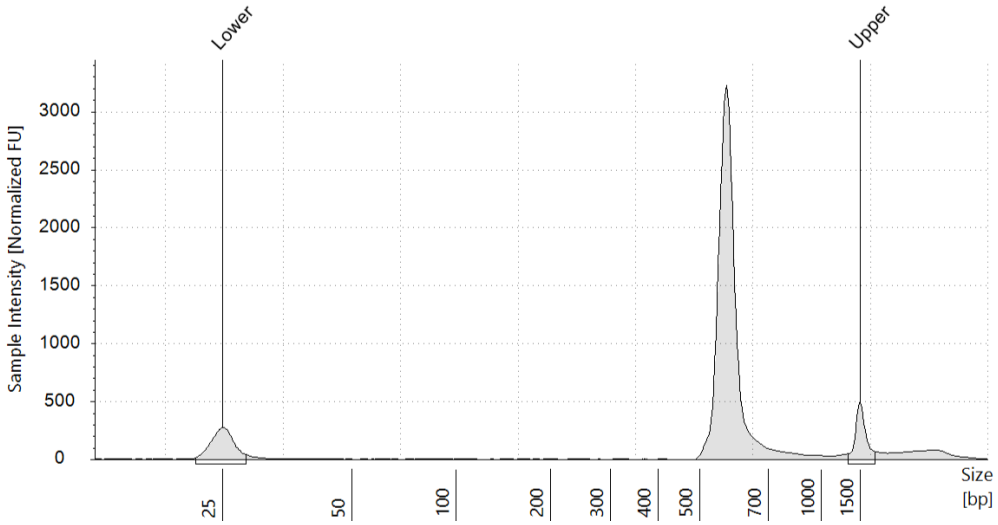
2.3.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 14 µl to a new PCR tube. If elution volume was adjusted in step 2.3.9, transfer volume should also be adjusted.

2.3.12. We recommend assessing cDNA amplicon (from Step 2.3.11) concentrations with a Qubit® fluorometer.

Note: Amplicons may also be run on a Bioanalyzer or TapeStation® to confirm ~550 bp size of amplicons. To run on a TapeStation, dilute amplicon 10-fold with 0.1X TE Buffer and run 2 µl on a DNA High Sensitivity ScreenTape®. (See Figure 2.3.12. below for example of amplicon size profile on a Bioanalyzer).

Samples can be stored at –20°C for up to a week.

Figure 2.3.12: VarSkip Short SARS-CoV-2 cDNA amplicons generated from 1,000 total viral copies.



Kit Components

NEB #E7626S Table of Components

NEB #	PRODUCT	VOLUME
E7651A	LunaScript RT SuperMix	0.048 ml
E7652A	Q5 Hot Start High-Fidelity 2X Master Mix	0.3 ml
E7725A	NEBNext ARTIC SARS-CoV-2 Primer Mix 1	0.042 ml
E7726A	NEBNext ARTIC SARS-CoV-2 Primer Mix 2	0.042 ml
E7657A	0.1X TE	1.3 ml
E7667A	Nuclease-free Water	1.5 ml
E8005A	NEBNext VarSkip Short SARS-CoV-2 Primer Mix 1	0.042 ml
E8006A	NEBNext VarSkip Short SARS-CoV-2 Primer Mix 2	0.042 ml

NEB #E7626L Table of Components

NEB #	PRODUCT	VOLUME
E7651AA	LunaScript RT SuperMix	0.192 ml
E7652AA	Q5 Hot Start High-Fidelity 2X Master Mix	1.2 ml
E7725AA	NEBNext ARTIC SARS-CoV-2 Primer Mix 1	0.168 ml
E7726AA	NEBNext ARTIC SARS-CoV-2 Primer Mix 2	0.168 ml
E7657A	0.1X TE	2 x 1.3 ml
E7667A	Nuclease-free Water	1.5 ml
E8005AA	NEBNext VarSkip Short SARS-CoV-2 Primer Mix 1	0.168 ml
E8006AA	NEBNext VarSkip Short SARS-CoV-2 Primer Mix 2	0.168 ml

Companion Products

NEB #	PRODUCT	VOLUME
T2010S	Monarch® Total RNA Miniprep Kit	50 preps

NEBNext ARTIC SARS-CoV-2 Primer Mix 1 and 2

NEBNext ARTIC SARS-CoV-2 Mix 1 and 2 for SARS-CoV-2 genome amplification are based on hCoV-2019/nCoV-2019 Version 3 (v3) sequences with balanced primer concentrations. Sequence information can be found at:
https://github.com/joshquick/artic-ncov2019/blob/master/primer_schemes/nCoV-2019/V3/nCoV-2019.tsv

NEBNext VarSkip Short SARS-CoV-2 Primer Mix 1 and 2

NEBNext VarSkip Short SARS-CoV-2 Mix 1 and 2 for SARS-CoV-2 genome amplification were designed to reduce the impact of variants on amplification efficiency. Sequence information can be found at:
https://github.com/nebiolabs/VarSkip

Checklist

1. cDNA Synthesis

[_] 1.1. Setup cDNA synthesis reactions, mix, and spin down:

- [_] 8 µl RNA Sample (OR Nuclease-free Water for no template control)
- [_] 2 µl LunaScript RT SuperMix

[_] 1.2. Thermal cycle (Heated lid to 105°C; 2 min 25°C, 20 min 55°C, 1 min 95°C, hold at 4°C)

2. Targeted cDNA Amplification

Note: Choose either the NEBNext ARTIC Primer Mix 1 and 2 or NEBNext VarSkip Short Primer Mix 1 and 2 before proceeding.

[_] 2.1.A. Setup, mix, and spin down targeted cDNA amplification reactions, Pool Set A:

- [_] 4.5 µl cDNA sample
- [_] 6.25 µl Q5 Hot Start High-Fidelity Master Mix
- [_] 1.75 µl SARS-CoV-2 Primer Mix 1

[_] 2.1.B. Setup, mix, and spin down targeted cDNA amplification reactions, Pool Set B:

- [_] 4.5 µl cDNA sample
- [_] 6.25 µl Q5 Hot Start High-Fidelity Master Mix
- [_] 1.75 µl SARS-CoV-2 Primer Mix 2

[_] 2.2. Thermal cycle (Heated lid 105°C; 98°C 30 sec, 35 cycles of 95°C for 15 sec and 63°C for 5 min, hold at 4°C).

[_] 2.3. Combine the Pool A and Pool B PCR reactions for each sample.

Note: Please consider the downstream application before proceeding. Section 3 lists instructions for a standard bead cleanup, however, it may not be necessary for your application.

3. Cleanup of cDNA Amplicons

[_] 3.1. Vortex beads

[_] 3.2. Add 20 µl of beads to sample and mix by pipetting 10 times

[_] 3.3. Incubate for 5 min

[_] 3.4. Place tubes on magnet

[_] 3.5. Wait 5 min and remove supernatant (keep the beads)

[_] 3.6. On magnet add 200 µl 80% ethanol, wait 30 seconds and remove

[_] 3.7. Repeat Step 3.6 once

[_] 3.8. Air dry beads, do not overdry

[_] 3.9. Off magnet add 16 µl 10 mM Tris-HCl or 0.1X TE. Elution volume may be adjusted for specific downstream applications

[_] 3.10. Mix by pipetting 10 times and incubate 2 min

[_] 3.11. Place tubes on magnet, wait 5 min, and transfer 14 µl to a new tube. If elution volume was changed in Step 3.9, the transfer volume should be adjusted accordingly.

[_] 3.12. Assess cDNA amplicon concentrations with a Qubit fluorometer

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	4/21
2.0	Create Chapter 1 and 2. Update protocols	9/21

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